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The neural stem cell/carnitine malnutrition hypothesis: new prospects for effective reduction of autism risk?

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Autism spectrum disorders (ASDs) are developmental neuropsychiatric disorders with heterogeneous etiologies. As the incidence of these disorders is rising, such disorders represent a major human health problem with escalating social cost. Although recent years witnessed advances in our understanding of the genetic basis of some dysmorphic ASDs, little progress has been made in translating the improved understanding into effective strategies for ASD management or minimization of general ASD risk. Here we explore the idea, described in terms of the neural stem cell (NSC)/carnitine malnutrition hypothesis, that an unappreciated risk factor for ASD is diminished capacity for carnitine-dependent long-chain fatty acid β-oxidation in neural stem cells of the developing mammalian brain. The basic premise is that fetal carnitine status is a significant metabolic component in determining NSC vulnerability to derangements in their self-renewal program and, therefore, to fetal ASD risk. As fetal carnitine status exhibits a genetic component that relates to *de novo* carnitine biosynthesis and is sensitive to environmental and behavioral factors that affect maternal circulating carnitine levels, to which the fetus is exposed, we propose that reduced carnitine availability during gestation is a common risk factor that lurks beneath the genetically complex ASD horizon. One major prediction of the NSC/carnitine malnutrition hypothesis is that a significant component of ASD risk might be effectively managed from a public policy perspective by implementing a carnitine surveillance and dietary supplementation strategy for women planning pregnancies and for women in their first trimester of pregnancy. We argue that this prediction deserves serious clinical interrogation.

Autism spectrum disorders (ASDs)[2] are neurodevelopmental disorders characterized by core symptoms of impaired social interaction and communication and repetitive, restricted behaviors. Other deficits, such as intellectual disabilities and epilepsy, are also frequently on display. ASDs include autism, Asperger syndrome, and pervasive developmental disorder (not otherwise specified) and can be either dysmorphic or nondysmorphic. These disorders already affect 1.5%-3% of young children in the United States, Europe, and Australia. Moreover, ASD management extracts a heavy social cost. Current estimates are that the annual toll of ASD management in the United States alone will exceed $450 billion by the year 2025 (1, 2), and these estimates do not take into account the heavy emotional burden shouldered daily by caretakers of the afflicted. Unfortunately, ASD incidence, whether as a result of increased awareness/diagnosis or genuine increase in disease (3), is still climbing rapidly. Deciphering the mechanisms underlying ASDs and the associated risk factors is therefore an area of intense public and biomedical interest.

Although it is generally accepted that ASD etiology includes both genetic and environmental factors (4, 5), effective strategies for treating core symptoms of ASDs or, more importantly, for ASD prevention remain elusive. One major reason for this unwelcome state of affairs is that the foundational mechanisms that underlie these diseases and the associated risk factors are not resolved. Clearly, although our understanding of the genetic basis of ASDs has advanced rapidly in recent years, most studies focus on a limited number of molecular pathways that specify synaptic development and function (6–7). Where particularly impressive progress has been made is in the rarer cases of dysmorphic ASDs; that is, cases where an associated physical deformity offers a strong phenotype through which genetic causation can be reliably tracked and identified. For instance, intensive studies of fragile X syndrome identified specific molecular targets currently under interrogation in several ongoing clinical trials (8, 9). Unfortunately, the great majority of ASD cases are nondysmorphic; that is, cases whose diagnoses rely on behavioral phenotypes whose recognition at early stages is uncertain. In those cases, the genetic analyses invariably become more complicated. The available evidence reports that the genetic etiologies of ASDs and the associated risk factors are extremely heterogeneous. Present estimates suggest that these genetic components will ultimately number in the hundreds, and genetic risk factors implicated to date include genes encoding proteins that regulate diverse cellular functions, such as synaptic development and maturation, chromatin remodeling and transcription, cytoskeleton organization, cell cycle progression, and intermediary metabolism (4, 5, 10).

Nondysmorphic ASDs are typically diagnosed when children are 2–4 years of age because these syndromes are recognized on

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[2] The abbreviations used are: ASD, autism spectrum disorders; NSC, neural stem cell; FAO, fatty acid β-oxidation; E11.5, embryonic day 11.5; IPC, intermediate progenitor cell; MCFA, medium-chain fatty acid; LCFA, long-chain fatty acid; VLCAD, very-long-chain acyl-CoA dehydrogenase; TCA, tricarboxylic acid; TMLHE, trimethyl-lysine hydroxylase; EGFP, enhanced GFP.

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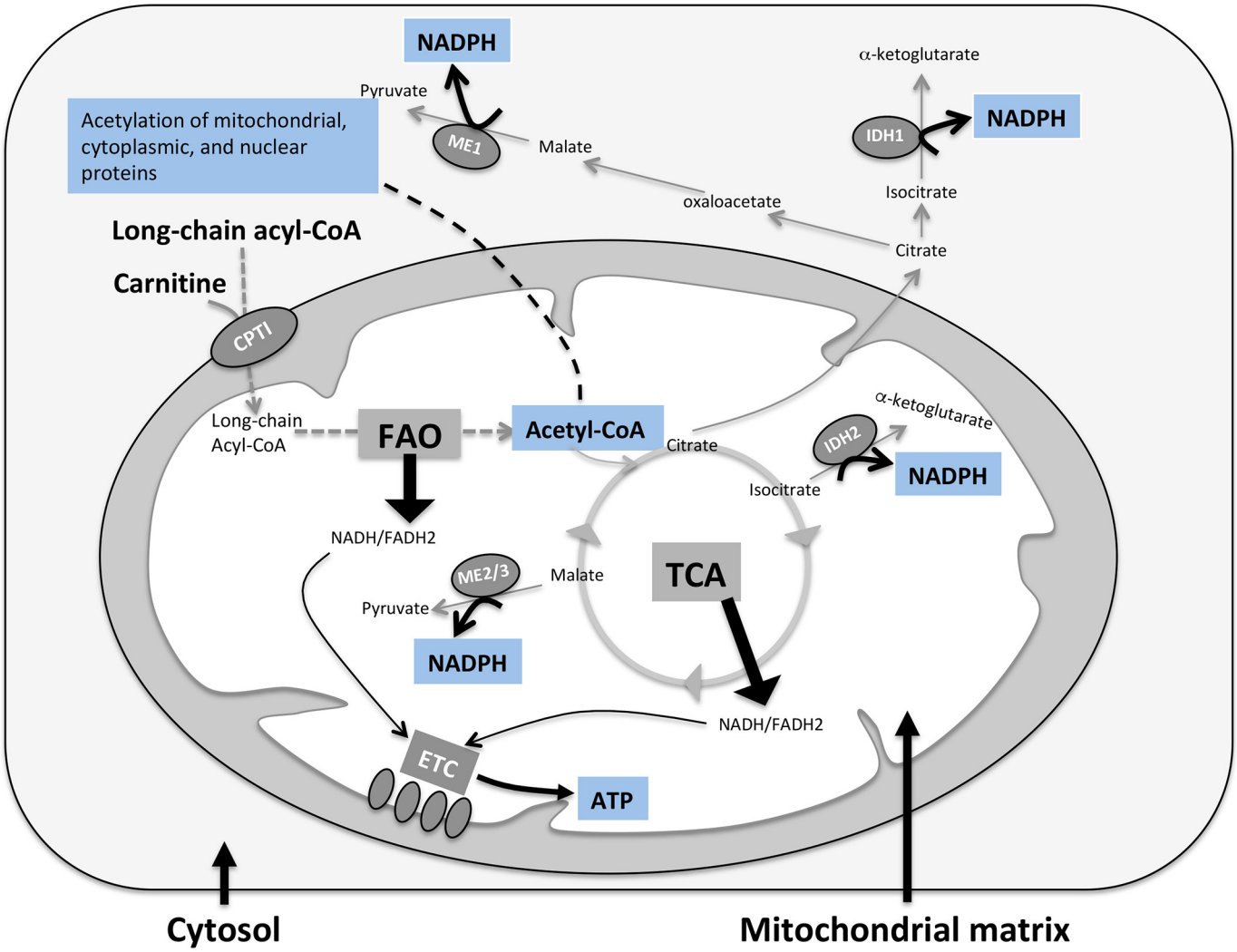

Figure 1. Carnitine-dependent long-chain fatty acid β-oxidation produces ATP-reducing power and acetyl-CoA. Carnitine-activated long-chain fatty acids (*long-chain acyl-CoA*) are transported into the mitochondrial matrix for β-oxidation. This import reaction is initially catalyzed by the carnitine acyltransferase CPT1, followed by the actual transport step and, finally, reconversion of the acyl-carnitine to acyl-CoA (not shown). Within the mitochondrial matrix, long-chain acyl-CoA undergoes multiple cycles of β-oxidation to generate acetyl-CoA, which then enters the tricarboxylic acid (TCA) cycle or serves as donor of acetyl groups for posttranslational modification of proteins. Both FAO and TCA cycle activities generate the reduced coenzymes NADH and FADH2, which are consumed in ATP production via the electron transport chain (*ETC*). By supplying acetyl-CoA to the TCA cycle, β-oxidation increases generation of the TCA intermediates isocitrate and malate in mitochondria and the cytoplasm. Isocitrate and malate are subsequently used as substrates by isocitrate dehydrogenases (*IDHs*) and malic enzymes (*MEs*), respectively, to generate reducing power in the form of NADPH both in mitochondria and in the cytoplasm.

the basis of behavioral/social phenotypes (11). Thus, the condition has already taken root by the time it is diagnosed, and subsequent intervention necessarily falls into the ASD management category. This basic reality poses several key questions that have not been directly addressed. First, at what precise stage is ASD risk first imposed? Effective prevention strategies require an answer to this question. Second, are ASDs truly a collection of hundreds of diseases? If so, prevention strategies reduce themselves to what are essentially case-by-case approaches. Rapid progress under such circumstances would seem rather hopeless. But what if there is a major risk factor common to a significant fraction of the population that lurks unappreciated beneath the phenotypic horizon, a common risk factor that, when combined with any one (or any other combination) of a heterogeneous cohort of other risk factors, strongly escalates the likelihood of ASD via a coincidence-reinforcement mechanism? If so, then the prospects of reducing ASD risk via a prevention regime improve dramatically when that common risk factor is identified. We are now encouraged to think that this might be the case.

Here we explore the idea that a specific form of embryonic neural stem cell (NSC) malnutrition for the amino acid derivative carnitine forms a substantial foundation for increased ASD risk. Carnitine is a micronutrient required for import of long-chain fatty acids into mitochondria for the purpose of fueling the critical metabolic pathway of fatty acid β-oxidation (FAO) (Fig. 1). FAO is not only a potent engine for ATP production but also a robust machine for generating reducing power and producing acetyl-CoA, a versatile intermediary metabolite (12, 13). The idea that NSC carnitine malnutrition defines a common ASD risk factor holds the attractive feature of suggesting new strategies for mitigating ASD risk in the general population by routine prenatal carnitine supplementation during pregnancy. This strategy, should it pass clinical muster, would not only be

amenable to rapid deployment in the public sector, as carnitine is a natural product already in public use, but it falls under the umbrella of ASD prevention strategies rather than measures for disease maintenance.

Prenatal neocortical development

ASDs are commonly diseases of the forebrain. One region of the forebrain, the neocortex, houses the intricate circuitry responsible for higher-order functions such as perception, cognition, language, and behavior. This region of the mammalian brain is a laminated structure with distinct neuronal cell types populating distinct layers. The majority of neurons (70%–80%) in the neocortex are excitatory pyramidal neurons. These cells are derived from dorsal forebrain NSCs via a prenatal developmental program (14). NSCs are unusual bipolar neuroepithelial and radial glial cells that span the entire neocortex, from the ventricular surface to the cortical plate, throughout development. During neurogenesis, NSCs proliferate rapidly to produce distinct sets of neuronal cell populations in a temporally precise manner. The first birth wave produces neurons that migrate and ultimately occupy the deepest layer of the cortical plate. Neurons born later migrate past the early-born neurons and populate superficial layers of the cortical plate.

The neocortex of mouse embryos is the most commonly exploited model for studying NSC homeostasis and neurogenesis during mammalian development. NSCs of the developing mouse neocortex give birth to neurons in the window of embryonic day 11.5 (E11.5) to E17.5. Although NSCs can divide to produce neurons directly or indirectly through different populations of lineage-restricted intermediate progenitor cells (IPCs), most neurons are produced indirectly through an IPC population (15, 16). These IPCs generally undergo symmetric divisions to produce two IPCs or two neurons. Before neurogenesis occurs, however, NSCs divide symmetrically to self-expand. During neurogenesis, NSCs modify their division program to accommodate three types of divisions: symmetric self-expanding divisions to produce two NSCs, asymmetric divisions to produce one NSC and one daughter cell at more differentiated stage (*i.e.* either IPC or neuron), and symmetric differentiating divisions to produce two daughter cells at a more differentiated stage (*i.e.* either IPC or neuron).

Exquisitely balanced control of NSC homeostasis is crucial for generating the correct numbers and types of neurons in the developing brain (14). Inappropriate depletion of NSCs by any one of a number of mechanisms penalizes production of later-born classes of neurons with resulting imbalances in types and numbers of neurons. Even when subtle, these imbalances are expected to enforce derangements in neuronal circuitry that result in development of a functionally compromised neocortex. In that regard, several lines of evidence implicate NSCs in ASD pathogenesis by this general pathway. First, a number of ASD risk genes encode modulators of chromatin structure that are established or suspected regulators of cell proliferation and/or stem cell self-renewal. Interestingly, the ever expanding list of ASD risk factor candidate genes overlaps significantly with analogous lists of cancer risk genes, an overlap united by the fact that the shared genes are typically drivers of cell proliferation (17). Second, the traditional ASD-linked genes/pathways (Wnt, PTEN, TSC1/TSC2, FMR1) also impinge on NSC self-renewal and differentiation during development (18–21), although these activities in the context of NSC biology are largely ignored in contemporary discussions of mechanisms of ASD pathogenesis. Third, MRI and postmortem histological studies identify cerebral cortex anatomical abnormalities in the brains of ASD-afflicted patients that are consistent with deranged neuronal production from NSCs during fetal development (22, 23).

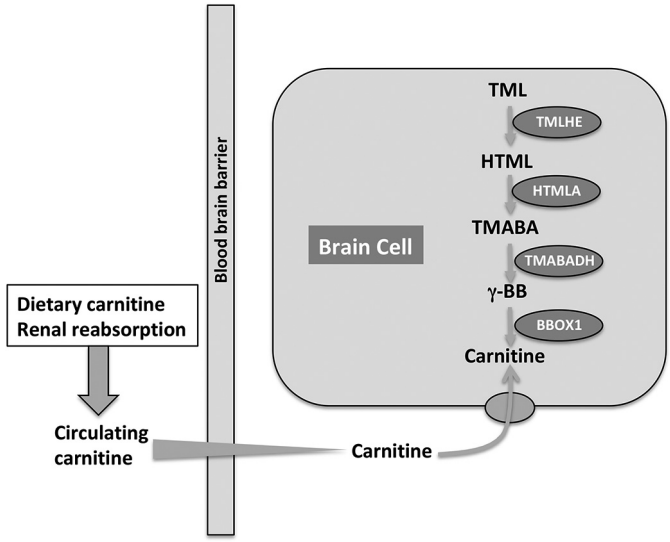

Figure 2. Carnitine homeostasis in the brain. Brain cells synthesize carnitine *de novo* and also import exogenous carnitine from interstitial cell fluid. Under normal conditions, the levels of circulating carnitine are primarily set by dietary carnitine intake and renal carnitine reabsorption. *De novo* biosynthesis of carnitine is limited in mammals. The process initiates with trimethyllysine (*TML*) and involves four biochemical reactions sequentially catalyzed by trimethyl-lysine hydrolase ϵ (TMLHE), hydroxyl trimethyl-lysine aldolase (*HTMLA*), trimethylaminobutyraldehyde dehydrogenase (*TMABADH*), and γ-butyrobetaine hydroxylase 1 (*BBOX1*), respectively.

TMLHE mutations are linked to ASD risk

A hallmark feature of ASD is the predisposition of males to these disorders, with a male:female ratio approaching 4:1 (24). This sex-linked susceptibility has focused searches for X-linked mutations that show genetic associations with ASDs. It is from this perspective that we first focus on the X-linked gene *TMLHE* and its association with ASD risk from the perspective of genome sequencing studies. These studies form a major pillar of our hypothesis that suboptimal fatty acid β-oxidation capacity, when combined with other dietary or environmental factors, substantially elevates ASD risk for the developing fetus.

TMLHE encodes a trimethyl-lysine hydroxylase that catalyzes the first of four biochemical reactions involved in carnitine biosynthesis (Fig. 2). Exon-focused array comparative hybridization studies identified deletion of exon 2 of the *TMLHE* gene in a male ASD proband (25), and subsequent work demonstrated the deletion to result in severe deficiencies in TMLHE enzyme activity and be enriched in probands from male–male multiplex ASD families relative to control males (26). Additional genetic evidence of a link between *TMLHE* deficiencies and ASD risk was obtained from X chromosome exome analyses, which identified a *TMLHE* nonsense mutation

(c.229C>T/p.Arg77X) that segregated with autism in one of 12 families analyzed (27). Moreover, interrogation of the *TMLHE* coding sequence from 501 unrelated male ASD probands identified two additional missense mutations (c.730G>C/p.Asp244His and c.1107G>T/p.Glu369Asp) that were absent in 303 healthy male controls. A case control study linking rare complete gene knockouts on autosomal and X chromosomes to ASD risk independently identified a hemizygous *TMLHE* loss-of-function mutation in one of 1245 male ASD probands but not in 899 male controls (28), and a frameshift mutation resulting in impaired TMLHE enzyme activity was identified in a male child with regressive ASD (29).

Other evidence also points to deficiencies in carnitine biosynthesis resulting in ASD-type disorders. For example, BBOX1 catalyzes the last step of carnitine biosynthesis (Fig. 2), and a young female who presented microcephaly, speech delay, growth retardation, and minor facial anomalies was found to carry a homozygous deletion of *BBOX1* (30). Straightforward interpretation of this patient is complicated, however, as deletion of a second gene (*FIBIN*) was also detected.

TMLHE deficiencies are high-incidence inborn errors of metabolism

Although inborn errors of metabolism were thought previously to be rare disorders, with an estimated incidence for any one disorder at a frequency of 1 in 10,000 or lower (31), the prevalence of TMLHE deficiencies is far more common than that of any previously known inborn error of metabolism. For example, the *TMLHE* exon 2 deletion allele was detected at a frequency of 1 in ~350 in control males in one study (26) and 1 in ~900 control males in an independent analysis (27). Moreover, mining of the Exome Aggregation Consortium database, comprised of exome sequencing data for 60,706 unrelated individuals without severe pediatric diseases (33,644 males and 27,062 females), confirmed that deleterious *TMLHE* point mutations and small insertion/deletions are remarkably common in the normal population (incidence of at least 1 in ~500 male individuals (32)). These are truly startling numbers. Thus, *TMLHE* satisfies important criteria for a major ASD risk factor that is common to a significant fraction of the population but lurks beneath the phenotypic horizon: TMLHE is associated with autism-risk, the gene is X-linked, and mutations in *TMLHE* are remarkably frequent in the normal population. The remarkably high incidence of *TMLHE* deficiencies in seemingly healthy individuals can be interpreted as evidence that such deficits present a low-penetrance ASD risk. However, as discussed below, we argue that penetrance considerations based on genetic data alone badly underestimate the association between carnitine availability, fatty acid β-oxidation capacity, and ASD risk.

NSC homeostasis requires carnitine biosynthesis and long-chain FAO

Our interest in *TMLHE* derived not only from the high incidence of mutations circulating in the human population but also from the fact that this X-linked gene is robustly expressed in the ventricular zone of the developing mouse neocortex (33); that is, the region of embryonic brain where the somata of NSCs

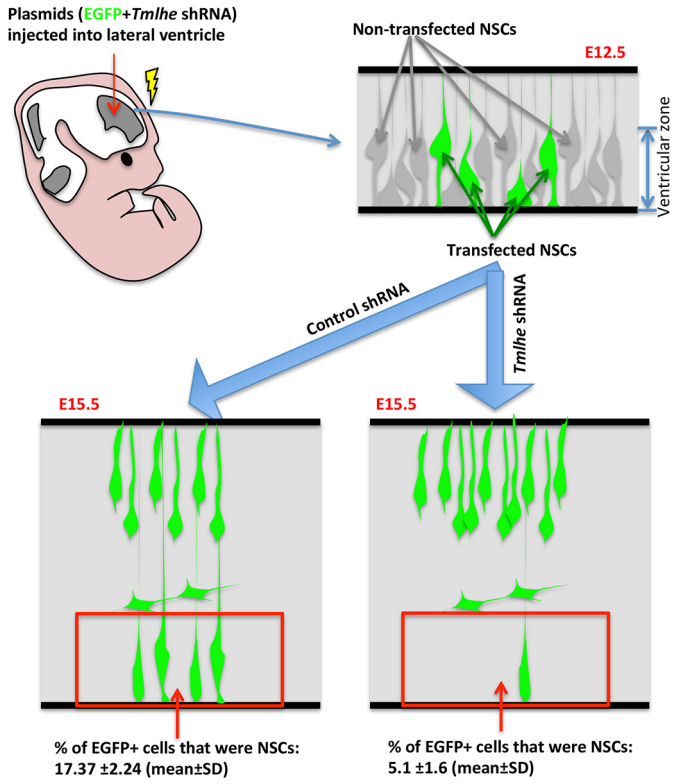

Figure 3. *In utero* electroporation as an approach to interrogate TMLHE function in NSCs. In a survival surgery procedure performed on a pregnant mouse, a mixture of plasmids (for expressing EGFP and either control or *Tmlhe* shRNA) is injected into the lateral ventricle of the mouse embryo. Short electric pulses are delivered across the head of the embryo to induce uptake of the plasmids by cells with access to the lateral ventricle (predominantly NSCs, which extend a basal process to the pial surface and an apical process to the ventricular surface). Electroporated embryos are returned to the uterus and allowed to incubate *in utero* for an appropriate period dictated by the experiment (72 h in this case), during which transfected NSCs divide to self-renew and produce differentiated cells (IPCs and neurons). Embryos are subsequently sacrificed, and confocal imaging analyses of fixed and immunostained brain sections are executed to fate the EGFP$^+$ cells as either NSCs or IPCs (numbers indicated in the figure (33)). The fractional representation of NSCs was significantly reduced in the Tmlhe knockdown group compared with the control shRNA group. In this assay, only cell-autonomous effects are monitored, as the transfected NSCs are surrounded by nontransfected NSCs and therefore reside in an unperturbed neurological niche. Interference with carnitine synthesis, with mobilization of fatty acids from lipid droplet stores, or with import of acyl-carnitines from the cytoplasm into mitochondria consistently results in depletion of NSCs from the ventricular zone of the developing neocortex (33).

that fuel development of the neocortex reside (14). This raises the question of whether TMLHE deficits and, by extension deficiencies in fatty acid β-oxidation, perturb development of the embryonic neocortex *in vivo* via disturbance of NSC activities.

To examine this question in a system that preserves the complex neurological niche in which NSCs reside, *in utero* electroporation technology was deployed to impose TMLHE deficits in individual NSCs in the neocortex of mouse embryos (34). The basic outline of the experimental design is illustrated in Fig. 3. It should be noted that the *in utero* electroporation approach interrogates cell-autonomous effects of TMLHE depletion in individual NSCs under conditions where nonautonomous factors provided by the neurological niche remain unperturbed. Using this experimental regime, shRNA-induced diminutions in TMLHE expression were imposed upon NSCs at an early stage of neurogenesis (E12.5). Such interventions resulted in a

significant depletion of the NSC pool within 72 h, with no obvious compromise in neuronal migration or differentiation. Rescue experiments demonstrated that NSC depletion was rescued by coexpression of WT TMLHE but not by expression of the catalytically dead TMLHE^{D244H} (34), *i.e.* a mutant that has been identified previously in a human ASD proband (26). Moreover, the NSC depletion phenotype was independently recapitulated by acute knockdown of carnitine acyltransferase (CPT1A) expression (*i.e.* the rate-limiting enzyme for FAO that produces the long-chain acyl carnitine that is imported into mitochondria), specific inactivation of CPT1A with the small-molecule inhibitor etomoxir, and expression of a dominant-negative form of perilipin 1 that inhibits fatty acid mobilization from lipid droplets for subsequent FAO (33). These collective data established that interference with FAO activity at several independent points consistently results in depletion of FAO-deficient NSCs from the developing embryonic neocortex and that this failure represents a cell-autonomous defect. That is, even a healthy neurologic niche is unable to supply sufficient exogenous carnitine to FAO-deprived NSCs to rescue self-renewal, indicating that endogenous NSC carnitine biosynthesis is an important factor in maintaining biologically sufficient FAO activity. This result is consistent with measurements reporting that the carnitine concentrations in cerebrospinal fluid are at least 10-fold lower than those in serum (35, 36).

There are a variety of mechanisms by which NSC depletion could occur, and a number of these have been experimentally excluded. FAO deficiencies do not predispose the affected NSCs to apoptosis, nor do FAO deficiencies derange other key cell biological features of NSCs, such as apicobasal polarity, interkinetic nuclear migration, cleavage plane orientation, or cell cycle parameters (34). Rather, TMLHE deficits induce depletion of NSC pools by disturbing the exquisitely balanced NSC self-renewal division program designed to produce cells fated to differentiate to neurons while preserving the NSC pool (34). As described above, NSC cell division occurs primarily via an asymmetric self-renewing mode where one daughter cell is an NSC and the other a restricted lineage cell (IPC) that is fated to differentiate into neurons (Fig. 4*A*). The two minor modes of NSC self-division are symmetric divisions. In one case, an NSC produces two NSC daughters, and in the other, the mother NSC produces two IPC daughters. The former is an NSC-expanding division mode, whereas the latter represents an NSC depletion mode.

Two independent assays were developed to analyze the products of individual NSC divisions in the embryonic mouse neocortex *in vivo* as a function of TMLHE activity (34). Both assays are founded on the principle of using *in utero* gene transfer to silence TMLHE expression in individual NSCs and to subsequently fate the two daughters produced by single cell divisions to identify which mode was followed in each division. Quantification of many such single divisions identified the distribution of cell division modes (Fig. 4*A*). The first assay employed a two-step *in utero* electroporation scheme to identify daughter NSC pairs (Fig. 4*B*), whereas the second assay used an *in utero* transfection scheme that reduced the efficiency of plasmid incorporation into NSCs but facilitated identification of isolated daughter NSC pairs for analysis (Fig. 4*C*). Both assays consistently demonstrated that TMLHE deficits significantly increase the frequency of symmetric NSC-depleting divisions at the expense of symmetric and asymmetric NSC self-renewing divisions (Fig. 4*D*) (34). NSC depletion was rescued by expression of WT TMLHE, but expression of the catalytically deficient TMLHE^{D244H} was completely ineffective in that regard (Fig. 4*D*) (34). Thus, TMLHE catalytic activity promotes NSC self-renewing cell division by inhibiting symmetric NSC-depleting divisions that produce two IPC daughters. Interestingly, this imbalance in NSC division mode induced by TMLHE deficiencies is fully carnitine-remedial in both *ex vivo* brain hemisphere culture systems (34) and *in vivo* when pregnant female mice are offered dietary carnitine supplementation.[3] The latter result is particularly relevant, as the pregnant mice in these experiments were maintained on a chow diet, which is essentially a vegetarian low-carnitine diet. Issues of diet will be addressed further below.

As would be expected given the phenotypes of human *TMLHE* loss-of-function mutants, *bbox1*-null mice are born alive and are nondysmorphic from the perspective that these animals exhibit anatomically normal brains with perhaps a very mild microcephaly at best.[3] That result implies the existence of compensatory mechanisms whose activities can modulate the developmental phenotype. In that regard, β-oxidation of medium-chain fatty acids (MCFAs) is a biochemically redundant pathway, and mitochondrial import of MCFAs is carnitine-independent (37). Thus, medium-chain FAO activity is one such candidate for a compensatory mechanism. Indeed, we found that inactivation of medium-chain FAO strongly exacerbates the self-renewal deficits of carnitine-deficient NSCs.[3] In that regard, the carrier frequencies of medium-chain FAO deficiency alleles in populations of Northern European origin are estimated to be as high as ~1 in 50 (38). That is, heterozygotes for these autosomal-recessive deficiencies are extremely common in the general population and provide yet another genetic factor linked to FAO deficiency with the potential to contribute to ASD risk.

TMLHE and NSC self-renewal

The collective data suggest a key role of carnitine and mitochondrial FAO for NSC homeostasis in the developing embryonic brain. What is the mechanism behind such regulation? FAO is a potent engine for both ATP production and generation of reducing power. Thus, *in utero* electroporation experiments were designed that employed fluorescent biosensor strategies for measuring ATP levels and redox status in the cytosol or mitochondrial matrix in unperturbed NSCs and in NSCs depleted of TMLHE or CPT1A (34). Although FAO defects resulting from either diminutions in TMLHE or CPT1A activity had no effect on ATP levels in either of the two compartments, the mitochondrial matrix of FAO-deficient cells was oxidized relative to the mitochondrial matrix of FAO-proficient NSCs (34). One interpretation of those results is that FAO deficiencies derange an NSC fate switch that responds to the mitochondrial redox state. In that regard, previous studies with NSCs of adult brain and NSCs cultured *ex vivo* in neuro-

[3] V. A. Bankaitis and Z. Xie, unpublished data.

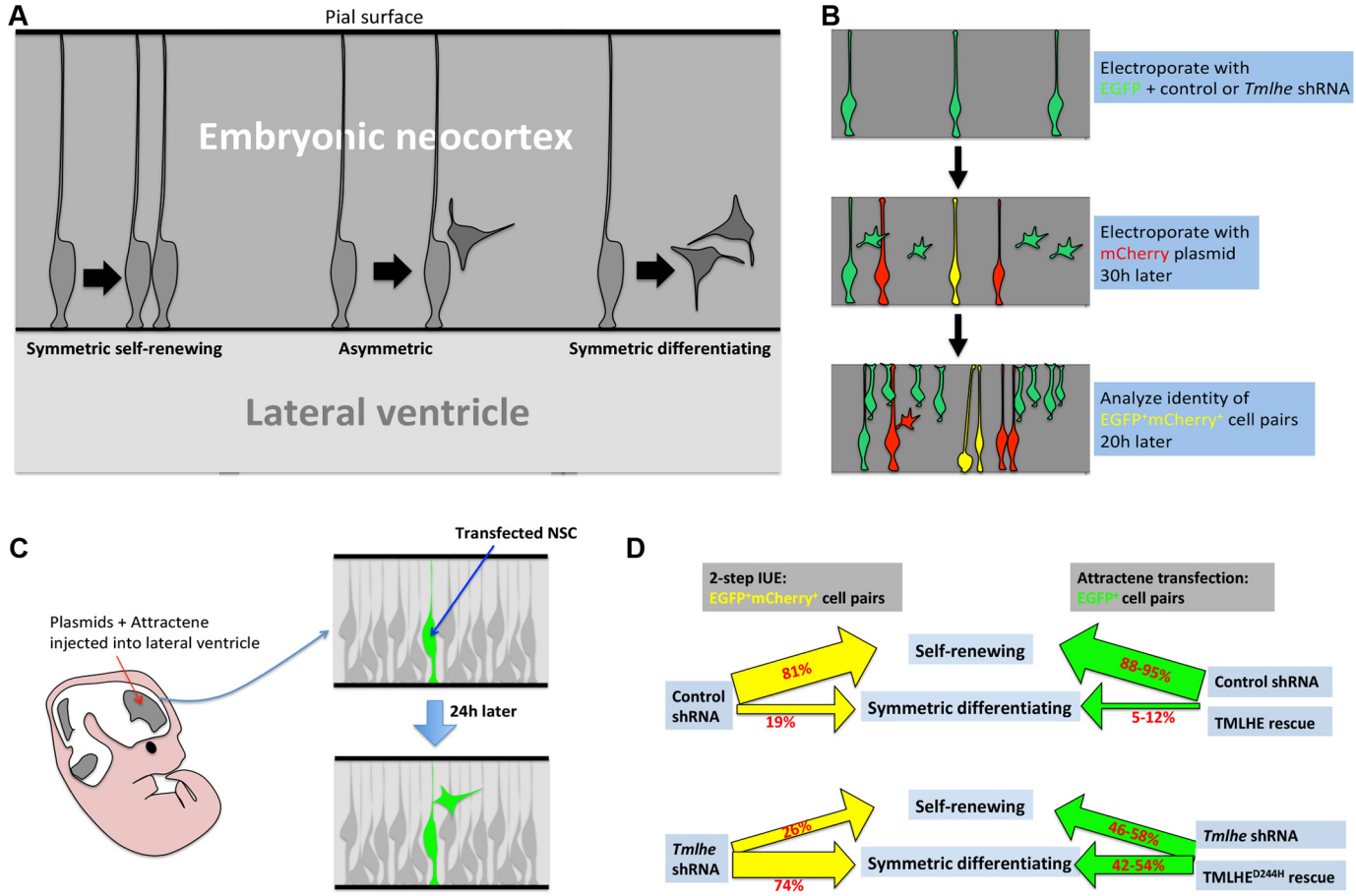

Figure 4. TMLHE deficiencies impair the NSC self-renewal program. *A*, modes of NSC divisions. NSCs generate neurons primarily through TBR2$^+$ IPCs. Only this route of neurogenesis is illustrated in the figure. Bipolar and multipolar cells in the figure represent NSCs and TBR2$^+$ IPCs, respectively. During neurogenesis, NSCs undergo three types of divisions: symmetric self-renewing divisions to generate two NSCs, asymmetric divisions to generate one NSC and one IPC, and symmetric differentiating divisions to generate two IPCs. *B*, two-step *in utero* electroporation assay for analyzing individual NSC divisions (33). EGFP$^+$mCherry$^+$ cell pairs are well isolated from each other and therefore can be confidently identified as two daughter cells derived from the division of a common mother NSC. *C*, Attractene transfection strategy for analyzing individual NSC divisions (33). Coinjection of the plasmid solution with Attractene reagent without electroporation leads to low transfection efficiency of NSCs lining the lateral ventricles. Thus, from a single transfected NSC (cell fate analyzed 24 h after DNA/Attractene injection), the adjacent pair of EGFP$^+$ daughter cells from the same initially transfected NSC can be confidently identified. D, the results obtained from the two experimental regimens were similar (33). In both assays, the vast majority of NSC divisions in control groups (control shRNA or *Tmlhe* shRNA coexpressed with shRNA-resistant WT TMLHE) were self-renewing, with at least one daughter cell as NSC. Under conditions of TMLHE deficiency (*Tmlhe* shRNA with or without coexpression of the TMLHE^{D244H} mutant), nearly half of the divisions exhibited by TMLHE-deficient NSCs were symmetric differentiating divisions that lead to stem cell depletion.

spheres suggest that FAO is important for maintaining both NSC ATP levels and the mitochondrial redox state (39, 40). Given the generally detrimental role of oxidative stress in stem cell self-renewal (41) and implications that oxidative stress is associated with ASDs (42, 43), the idea that mitochondrial oxidative stress caused by FAO deficiencies induces NSC differentiation is an interesting one and merits further investigation.

That mitochondrial FAO is a potent generator for acetyl-CoA also deserves consideration (Fig. 1). This intermediary metabolite is a versatile molecule that not only enters the tricarboxylic acid cycle but is also the direct donor of acetyl groups for protein acetylation; that is, a class of posttranslational modifications that have wide-ranging effects on protein activities, including activities of histones that are central players in epigenetic regulation of gene expression. Our preliminary results in the murine model support a contribution by such an epigenetic mechanism to maintenance of NSC homeostasis in the developing mammalian neocortex.[3] In that regard, it is of interest that the switch from solitary to gregarious social behavior of several locust species is epigenetically controlled and that carnitine is a key promoter of that behavioral switch (44). Thus, carnitine and FAO-fueled production of acetyl-CoA might power an epigenetic pathway conserved from insects to mammals for regulating behavior.

Human ASD and carnitine deficiency

Our studies of the contribution of FAO to NSC biology deploy a system where the physiological context of the manipulated NSCs is fully preserved, but the system comes with the caveat that individual NSCs are monitored in an essentially WT neurological niche. This is not the case in TMLHE mutant ASD patients, of course, where all NSCs in the tissue would be mutant. Furthermore, the studies described above were performed in mice. These issues raise the question of whether fetal carnitine and FAO deficiencies have any relevance to human ASD risk. Indeed, there is evidence to support a genuine association.

With regard to FAO, linkage of long-chain fatty acid (LCFA) β-oxidation deficits and ASD is suggested by neurodevelop-

mental assessments of children with inborn errors of fatty acid β-oxidation regardless of fatty acid chain length (45). Very-long-chain acyl-CoA dehydrogenase (VLCAD) is required for β-oxidation of LCFAs in human tissues and, among the 14 children with VLCAD deficiencies, four exhibited speech delay or language weakness, and one displayed ASD behavior. Of the two children with deficiencies in long-chain 3-hydroxyacyl-CoA dehydrogenase, another enzyme required for β-oxidation of LCFAs in human tissues, one exhibited speech delay, and the other was diagnosed with pervasive developmental delay consistent with ASD. Independent analysis of seven children identified by newborn screening as VLCAD-deficient identified one as high on an autism spectrum subscale, and another was formally diagnosed with ASD (46). In patients diagnosed with long-chain 3-hydroxyacyl-CoA dehydrogenase deficiencies prior to newborn screening, an increased incidence of ASDs has also been observed (47).

Carnitine deficiencies in ASD patients were first recognized some two decades ago (48, 49) and later confirmed in several independent studies that enrolled significant patient cohorts. In retrospective analyses of serum metabolites of 100 ASD children, 83% were found to present total and free carnitine levels below the reference mean (50). Moreover, the levels of total carnitine and free carnitine were equal to or greater than one standard deviation below the reference mean in 36% and 27% of these ASD children, respectively (50). Another report comparing 100 ASD children with 100 healthy controls documented reduced serum carnitine levels in 66% of the ASD children (51). Although all of those studies involve children, and our mouse studies document NSC derangements in imposed in mid-gestation, the clinical data are nonetheless consistent with the carnitine deficiency hypothesis. In that regard, multiple studies document significant decreases in maternal serum carnitine levels during pregnancy that approach pathologically low levels (52–59). Interestingly, that decline in carnitine levels commences at the onset of mid-gestation (12 weeks, 58,59), a window that corresponds to the one where we interfere with carnitine synthesis and fatty acid β-oxidation in our mouse *in utero* electroporation experiments (34). Although the functional significance of those associations with regard to ASD risk has not been interrogated, those data do suggest that maternal circulatory carnitine is often an unreliable source of this micronutrient to the developing fetal brain.

TMLHE mutations and the phenotypic penetrance paradox

Although *TMLHE* is an ASD-linked gene, contemporary thought holds that the linkage is weak because the penetrance of *TMLHE* mutations is poor. The argument to this effect comes from genome sequencing data that, while identifying *TMLHE* mutations as high-incidence inborn errors of metabolism, also indicate that TMLHE-deficient humans do not typically present ASD symptoms (26, 27). Based on the frequency of the *TMLHE* exon 2 deletion in male–male multiplex ASD families, the phenotypic penetrance of *TMLHE* mutations is estimated to be an unimpressive ~3% (26). However, genomics-based arguments assume that genetic status will accurately translate to circulating carnitine status. In our view, this base presumption is unjustified, as it ignores common behavioral and environmental factors that will independently influence maternal carnitine status; that is, the major carnitine supply available to the fetus and the sole carnitine resource for the TMLHE-deficient fetus. We suggest that maternal behavior and environment play far larger roles in the regulation of NSC carnitine status in the developing human fetus than currently appreciated, and that these nongenetic influences confound direct extrapolation of the genomics data.

One argument to this effect comes from examination of the biochemistry underlying carnitine biosynthesis. The first and the last enzymatic reactions in the conversion of trimethyl-lysine to carnitine are catalyzed by the *TMLHE* and *BBOX1* gene products, respectively. Both enzymes are Fe-dependent dioxygenases. Carnitine biosynthesis is therefore sensitive to iron homeostasis. Clinical evidence to support this contention derives from studies where reduced serum carnitine levels are routinely detected in children suffering from iron deficiency anemias (60, 61). Regarding NSC carnitine malnutrition, fetal iron deficiency follows maternal iron deficiency, and this syndrome is common. Iron deficiencies afflict 30–50% of pregnant women in the United States, with higher incidence globally (61–65). Thus, this extremely common nongenetic condition potentially represents a far more significant pathway for reducing fetal NSC carnitine levels than the *TMLHE* mutations themselves. Indeed, epidemiological studies report that iron deficiency is associated with a substantially elevated autism risk (66, 67)

Behavioral factors also impact maternal and, therefore, fetal carnitine status. Although humans have the capacity to synthesize carnitine *de novo* from trimethyl-lysine, that capacity is limited. The primary reservoir of carnitine comes from the diet, where some 75% of the circulating carnitines in the healthy human body are derived from exogenous sources (68, 69). Although the trimethyl-lysine that fuels carnitine biosynthesis is abundant in plants, vegetables, fruits, fish, and dairy products, these foods are carnitine-poor dietary sources. Red meat, however, is a particularly rich carnitine source. For comparison, normalizing the data per unit mass, where carnitine equivalents in beef are set at 1.00, pork (0.3), fish (0.06), chicken (0.04), and milk (0.10) are inferior carnitine sources (68). Plants are much poorer sources. Mass spectrometry–based measurements indicate that plant carnitine levels are several orders of magnitude lower than those present in animals (70). Thus, maternal diet during pregnancy is a potentially significant behavioral variable that impacts the fetal NSC carnitine supply. As vegetarianism and veganism are ever more popular dietary practices, these behavioral factors also potentially represent frequent pathways for reducing fetal NSC carnitine levels. Indeed, clinical data report that vegetarian and vegan women present significantly lower plasma carnitine levels than women who eat a mixed diet (69, 71, 72).

In situ synthesis of carnitine is limited to several tissues in mammals (liver, kidney, brain, and perhaps intestine (68, 69, 73–75)). Renal reabsorption plays a particularly critical role in systemic carnitine homeostasis, as circulatory carnitines are vigorously salvaged by the kidney. This salvage activity is mediated via the high-affinity carnitine transporter OCTN2 encoded by the *SLC22A5* gene (69, 73). OCTN2 sets the renal saturation threshold for carnitine excretion at ~50 μM, a value that closely coincides with normal plasma carnitine concentra-

tions (25–50 μM). Thus, renal carnitine reabsorption is highly efficient when circulatory carnitine concentrations are low. Any pathogenic conditions during pregnancy (*e.g.* infection) or pharmacological treatments (*e.g.* valproate (76)) that significantly impair either carnitine salvage or transport could expose fetal NSCs to an elevated risk of reduced maternal carnitine supply. The consequence would be suboptimal FAO activity during a critical developmental window. Moreover, environmental intoxication with heavy metals such as zinc, lead, and cadmium is also associated with impaired Fe homeostasis (77). Exposure of a pregnant woman to high levels of heavy metals in drinking water or otherwise also carries the risk of impairing *de novo* carnitine biosynthesis.

We emphasize that genetic and environmental factors independent of carnitine status, but associated with mitochondrial FAO activity, are also relevant. Carnitine-independent β-oxidation of MCFAs is an example. One newborn screening study reported that 13 of 27 children identified as suffering from medium-chain FAO deficits presented language/speech delay or frank speech deficits, one child exhibited autistic behavior, and four other children exhibited developmental deficits related to motor function (45).

The NSC carnitine malnutrition hypothesis: implications and questions

The principal thesis of this article is that NSC carnitine malnutrition poses a major unappreciated risk factor for ASD. The foundations for this argument include the fact that X-linked *TMLHE* mutations are associated with ASD risk and have a remarkably high incidence in the population. The risk of NSC malnutrition is further compounded by environmental and behavioral factors that impinge on the NSC carnitine supply independently of genetic status. These include maternal iron deficiencies, kidney dysfunction, heavy metal intoxication, and lifestyle factors such as maternal diet. The experimental foundation for the NSC/carnitine malnutrition hypothesis comes from our study of the consequences of carnitine and FAO deficiencies in murine embryonic NSCs during neurogenesis (34). That murine NSC study raises interesting ideas and questions related to ASD risk. Four ideas deserve particular emphasis.

First, the detrimental effects of carnitine and FAO insufficiencies on NSC self-renewal are apparent in mice by E15.5, a developmental stage that translates to mid-gestation in humans. This conclusion projects that prevention strategies for ASDs of either this specific etiology, or those to which carnitine deficiencies contribute when combined with some other risk factor(s), will not be effective when these rely on behavioral diagnoses in young children. That timing of diagnosis and intervention is well behind the curve in terms of deploying any meaningful prevention strategy. Our mouse studies indicate that the seeds for NSC dysregulation (and, inferentially, ASD risk) are already sown by mid-gestation (34). In this regard, we find the well-documented decline in carnitine levels that commences at the onset of mid-gestation in pregnant women to be of particular interest (52–59).

Second, the data speak to limitations of sole reliance on prenatal genetic testing for *TMLHE* mutations when devising strategies for ASD prevention. The environmental and behavioral factors that can give rise to secondary carnitine insufficiencies likely far outstrip genetic factors in terms of significance. Routine monitoring of circulating carnitines in pregnant women via direct biochemical measurement is a superior solution. Prenatal genetic testing remains beneficial for alerting cases where a parent is recognized as a carrier of a *TMLHE* mutation or when the embryo is recognized as genetically deficient in *de novo* carnitine synthesis. In those cases, targeted monitoring of circulating maternal carnitines throughout pregnancy might be of value, as it is the FAO-deficient fetus that will be particularly vulnerable to diminutions in those metabolites.

Third, consideration of maternal diet as a behavioral factor of relevance to ASD risk has its own interesting implications. This hypothesis raises the question of whether adoption of vegetarian or more extreme vegan lifestyles by women of child-bearing age, especially by pregnant women, inadvertently raises the specter of ASD risk for the developing fetus. The biochemical rationale for this idea is that women embracing such lifestyles would be predisposed to reductions in circulating carnitine concentrations (69, 71, 72). This idea has demographic implications. It is an interesting conjecture that appropriately designed cross-correlation analyses will identify such behaviors (and, therefore, rising ASD incidence) to be concentrated in affluent and, most likely, socially progressive urban communities. There is some evidence to support this idea (78).

Fourth, with regard to demographics, the genomics studies that identified *TMLHE* mutations as frequently occurring inborn errors of metabolism were weighted heavily in favor of populations of European descent (26, 27). One extrapolation of those data are that *TMLHE* mutations were tolerated, and therefore fixed, in that population because red meat has represented a dietary staple in that part of the world for thousands of years. That is, genetic deficiencies in carnitine synthesis were tolerated in the European population because their effects were nutritionally complemented by a carnitine-rich diet. In this manner, the selection pressures that would have otherwise eliminated such mutations from the population were effectively removed.

What is the incidence of *TMLHE* mutations in other populations/races where red meat has not been such a central dietary staple over the course of human history? The selection pressures for appropriate FAO activity would have remained intact in those populations over the course of their history, with the result that *TMLHE* mutations would be strongly disadvantaged in those cohorts. Interrogation of this question is within reach of existing genome sequencing technology, and we are of the opinion that there is now a significant rationale for examining this issue in detail.

Implementable prospects for reducing ASD risk

When the nongenetic factors that impinge on carnitine homeostasis in the mother and fetus are considered in aggregate, we estimate that some 20%–30% of pregnant women in the United States might be exposing the developing fetus to a suboptimal carnitine environment (the frequency of iron deficiency alone meets this level (61–65)). Even an ASD penetrance of 3%–5% in such potentially at-risk pregnancies would account for a very significant fraction of ASD cases. Thus, the NSC/carnitine malnutrition hypothesis calls for clinical exploration

of what would be a direct and rapidly implementable strategy for mitigating a potentially major and unappreciated ASD risk factor; that is, designation of carnitine as a recommended daily supplement for pregnant women. Our finding that NSC self-renewal imbalances caused by carnitine/FAO deficiencies in mice are remediated by supplementation of the maternal diet with carnitine supports the case (34).[3] As carnitine is a natural product, one already available to the public over the counter, clinically approved implementation of this strategy would not require the expensive and time-consuming process of securing approval by the Food and Drug Administration. Obviously, daily recommended intakes need to be clinically determined, as carnitine is a precursor to trimethylamine *N*-oxide (which has been implicated in cardiovascular disease (79), and high carnitine doses can also have other undesirable consequences (29, 80). However, clinical management of a safe dosage for pregnant women does not seem to be a particularly complicated hurdle. Carnitine use in adults for improving brain function, blood pressure, and heart performance already indicates that daily intake of 2 g of carnitine is safe (81). Indeed, a daily dose of 500 mg of carnitine is sufficient to correct the carnitine decline experienced during pregnancy, with no reported ill effects for mother or to fetus (59). Moreover, with regard to fetal health, carnitine supplementation of parenterally fed babies born prematurely (<37 weeks old) has been practiced for decades with no obvious ill effects (82), suggesting that carnitine supplementation during pregnancy is very likely to be well-tolerated by the fetus.

What is there to lose from the public health perspective by investing serious clinical effort in investigating a broad-based carnitine supplementation strategy? Essentially nothing, and the data suggest that there is much to gain. A clinically approved carnitine supplementation program would be analogous to the position taken in 1992 by the Public Health Service to recommend a daily dose of 400 μg of folate for all women of childbearing age as a mechanism to mitigate neural tube birth defects. This recommendation was subsequently extended by a Food and Drug Administration decision in 1996 to require folate enrichments be added to a variety of grain products. Implementation of folate supplementation arguably represents the most successful public health policy enacted to date. Perhaps a carnitine supplementation program will prove to be similarly successful.

Carnitine and cognitive development in early childhood

Although the focus of our work so far has been on the consequences of carnitine/FAO deficiencies for embryonic NSC biology (34), and we posit that it is at the fetal stage where carnitine supplementation will first be effective as prevention strategy, there is evidence to suggest that carnitine also plays a role in postnatal brain development in young children (83). Another case in point involves a male child who carries a *TMLHE* mutation and who was diagnosed with ASD at 4 years of age (29). A daily oral carnitine delivery regimen resulted in significant improvements in several key behavioral milestones. Because of secondary gastric effects caused by the high carnitine doses delivered, the supplementation dose for this patient was cycled. Importantly, cognitive improvements cycled in phase with carnitine supplementation (29). This correlation lends confidence that the beneficial effects were directly related to carnitine supplementation and is suggestive of some postnatal benefits. A follow-up pilot study also provides supporting, albeit preliminary, data (80).

In that regard, the diets of newborns and young children during their first 18 months of life are typically low in carnitine content. Nursing is followed by introduction to solid food via fruit- and cereal-dominated diets. Perhaps simple adjustments, such as offering toddlers a small daily dose of meat broth, will deliver underappreciated benefits in potentiating cognitive development, particularly for children with inborn errors in carnitine biosynthesis and fatty acid β-oxidation. There is a growing body of evidence to suggest that such measures have merit (84, 85).

Also, with regard to potential postnatal benefits of carnitine supplementation, we note that the NSC/carnitine malnutrition hypothesis does not adequately account for the sex disequilibrium signature of ASDs. It would seem that environmental factors that result in secondary fetal carnitine deficiencies would predominate over X-linked genetic factors such as *TMLHE* mutations. Although sex-linked contributions emanating from hormonal differences might help account for the signature male bias in ASDs, an interesting idea has been proposed by Beaudet (84) that young males might be intrinsically reduced in the capacity to transport carnitine across the blood–brain barrier. This sex-linked deficit is suggested to be due to reduced expression of an amino acid transporter expressed from an X-linked gene, *SLC6A14*, that might escape X inactivation (84).

Conclusions

Finally, we are struck by the fact that two developments dominating public interest in contemporary news cycles detail the seemingly unrelated topics of the alarming rise of autism in young children and the damaging human health and planetary-scale environmental costs associated with cattle farming and consumption of red meat (86). The meteoric rise of companies promoting adoption of meatless mimetics of beef and chicken at major fast food outlets testifies to the rapidly growing societal appetite for reducing meat consumption. This philosophy is even rising to the level of circulation of scientific petitions exhorting world governments to unite in adopting global measures to restrict meat consumption (87). We now pose the question whether such emerging societal attitudes regarding nutrition and its environmental impact are on collision course with increased ASD risk. Food for thought, indeed.

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